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Evaluation of Endocrine Disrupting Chemicals in the Florida Coastal Pelagic Fish Complex Following the Deepwater Horizon Oil Spill Event

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Abstract

Following the BP Deepwater Horizon oil spill event, there was a critical need to assess the effects of the oil and dispersant chemicals on the coastal pelagic fish complex in the Gulf of Mexico and the Florida Straits. The objective of this study was to determine if spilled crude oil and dispersant chemicals have posed an ecological risk to the coastal pelagic fish complex through the detection of vitellogenesis. Crude oil containing polycyclic aromatic hydrocarbons (PAHs), dispersant chemicals and other estrogen-mimicking compounds are suspected to induce vitellogenin production in male and immature female fish, normally only produced by sexually mature females. Blood plasma and surface mucus were collected from wild-caught adult and juvenile males and females from as many representative coastal pelagic species as possible (including yellowfin tuna *Thunnus albacares* and swordfish *Xiphias gladius*). To create a control for this experiment, crevalle jacks (*Caranx hippos*) and lookdowns (*Selene vomer*) were injected with estradiol-17β (10 μg/g body weight) into the peritoneal cavity to induce vitellogenesis, regardless of sex or reproductive stage. The mucus and blood plasma of each injected fish was collected 7 to 11 days post-injection. Mucus and blood plasma samples of wild-caught and experimentally-injected fishes were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis, stained with a phosphoprotein specific fluorescent dye (Pro-Q Diamond®), and visualized through ultraviolet transillumination. Vitellogenin was visibly detectable in the mucus collected from the control-injected fish, suggesting a disruption in the endocrine system as a result of estrogen exposure (estradiol-17β). However, there were no elevated levels of vitellogenin detected in any wild-caught fish mucus or blood plasma samples, indicating no vitellogenesis. From this, we infer that there has been no detectable endocrine disruption to the sampled coastal pelagic fish complex in the Gulf of Mexico two years after the start of the spill.

Key Words: fish; vitellogenin; estradiol-17β; pelagic; endocrine disruption.

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Introduction

On April 20, 2010, the Deepwater Horizon (DWH) oil rig working on the Macondo oil field exploration well for the oil company BP exploded, leading to the death of eleven people. A fire continued to burn for 36 hours until the rig sank, leading to a sea-floor oil flow that continued for 87 days until it was successfully capped on September 19, discharging an estimated 210 million gallons of crude oil. A massive response and cleanup team consisting of floating booms, oil skimmer vessels and localized controlled burning were used to try to prevent the oil from spreading to beaches and estuaries. The recovery effort also deployed approximately 1.84 million gallons of Corexit™ oil dispersant, applied both at the surface and at the underwater wellhead. Underwater injection of dispersant led to large sub-surface oil plumes and oil still reached the coastline in Louisiana, Mississippi, Alabama, Florida and Texas, devastating many species of marine zooplankton, fish, birds and mammals at all stages of life (Barron, 2012; Muhling *et al*, 2012; Powers *et al*, 2013). This study was designed to evaluate the effects of oil and dispersant chemicals in the Gulf of Mexico following the DWH oil spill through detection of abnormal vitellogenin production in large pelagic fishes.

Sexually mature female fishes naturally produce vitellogenin (VTG) to create the nutritive proteins and lipids found in the yolks of their eggs (Mommsen and Walsh, 1988). Vitellogenin is a precursor glycolipoprotein produced in the liver during the main phase of oocyte growth. Female fish release estrogens (particularly 17β-estradiol) from the ovaries and into the blood stream where they are carried to various places in the body. In the liver, estrogens induce the production of VTG by estrogen-mediated regulation (Mouchel *et al.*, 1996). The VTG is then carried in the blood from the liver to the oocyte

surface, where it is imported into developing oocytes via endocytosis and cleaved into egg yolk proteins and lipids, later to be used by the developing embryo as food (Parks *et al.*, 1999). However, elevated amounts of estrogens, estrogen mimics, and other endocrine disrupting chemicals in water systems can induce abnormal VTG production in males and immature females (Jobling and Tyler, 2003). Male fish normally carry negligible amounts of estrogens in their blood, but will rapidly synthesize vitellogenin when exposed to environmental estrogens (Sumpter, 1995). According to Heppell *et al*. (1995), most male fishes possess functional copies of the VTG gene that allow it to be readily induced not only by endogenous estrogens, but also by exogenous estrogen-mimicking compounds (Tolar *et al*., 2001). This induction of vitellogenesis in males, particularly at the crucial embryonic development stage, can have serious adverse effects. In Gimeno *et al*. (1997), genetically male common carp (*Cyprinus carpio*) were exposed to 4-*tert*-pentylphenol (an estrogenic alkylphenol) during sexual differentiation and formed oviducts, the female passageway for ovaries. With such detrimental effects on the development of reproductive organs, entire populations of fish could potentially be at risk if broadly and equally exposed to exogenous estrogens during critical developmental life stages.

Estrogens and other endocrine disrupting chemicals (EDCs) are already constantly entering water systems by various methods. Some problems of particular concern are through sewage-treatment, paper mill, and agricultural effluents (Folmar *et al.*, 1996; Jobling *et al.*, 1998; Harries *et al.*, 1997; Metcalfe *et al.*, 2000). The majority of rivers in the United States have at least one sewage treatment plant emitting treated water, and the effluent in some cases can constitute the majority (as high as 80 to 90

percent) of the total river volume if the river is slow-moving, which often occurs near urban areas during periods of low rainfall (Sumpter, 1995). However, it remains unclear whether these estrogenic outputs are the result of domestic and industrial input pass-throughs via these sewage treatment locations or if the treated sewage is transformed into estrogenic chemicals during the degradation process. With the potential for large amounts of chemical loads into the environment, it is difficult to determine conclusively whether one specific chemical or a mixture of several different chemicals is the cause of endocrine system disruption, much less to what degree.

Of all the potentially impacted organisms, fishes are of particular concern due to their obligatory aquatic existence. They are constantly in contact with all water pollutants, and many chemicals are readily taken up through their gills, diet, and skin. The addition of estrogens and estrogen mimics may not only alter the natural reproductive cycle, but may also compete with natural estrogen binding sites (White *et al.*, 1994). Jobling and Sumpter (1993) noted that non-ionic surfactants and alkylphenols found in sewage treatment effluent were estrogenic and interfered with the natural 17-β estradiol binding sites, disrupting natural estrogen-mediated processes. The degree of interference, however, was dependent on the chemical composition of the non-ionic surfactants and the various products of their biodegradation, alkylphenols being the final product. As the surfactants degrade, they become less water-soluble and more lipophilic, making them more biologically available to aquatic organisms. Jobling and Sumpter (1993) speculate that because of the lipophilicity and persistence of alkylphenols, they are bioaccumulative and are likely to be more potent in much lower concentrations than

the original surfactants. These alkylphenols persist in the sludge and effluent from waste treatment plants and enter the lakes and rivers on which the plants reside.

With known evidence of endocrine disruption in freshwater fishes via exogenous estrogen exposure, particularly from sewage treatment effluent, the necessity to assess similar potential impacts on marine fishes in the event of a large input of EDCs is critical. In the marine environment, the largest inputs of endocrine-disrupting chemicals typically come from large oil spills and subsequent clean-up efforts. Although there has been research on EDC inputs into the ocean following some of the major oil spills of recent years (e.g., Barron *et al.*, 2003; Bue *et al.*, 1998), it has still been difficult to assess population-wide effects for many species potentially affected by the oil spill event. The world's oceans are much larger and more dynamic than freshwater lakes and rivers, making experiments much more difficult to execute and control.

Coastal pelagic fishes reside in the relatively shallow waters above the continental shelf and are notable for the fact that they regularly travel from inshore to offshore environments. For a few coastal pelagic species, such as the Atlantic bluefin tuna (*Thunnus thynnus*), the Gulf of Mexico is a vital breeding ground throughout the summer months, which coincide with the dates of the DWH oil spill event and subsequent cleanup efforts. Considering that many studies have proven that the developing embryonic stages of the life cycle are at the highest potential for impact from PAHs (polycyclic aromatic hydrocarbons) and EDCs (e.g., Carls *et al*., 1999; Heintz *et al*., 1999; Incardona *et al*., 2004), this could be detrimental for local populations that may be breeding within oil spill event areas. Polycyclic aromatic hydrocarbons are organic molecules that can be found naturally in the environment and also as an anthropogenic input, such as from the

burning and breakdown of fossil fuels. Carls *et al*. (1999) exposed Pacific herring (*Clupea pallasi*) embryos to dissolved PAHs from weathered Alaska North Slope crude oil collected from the Exxon Valdez spill and found malformations, genetic damage, reduced swimming ability and mortality, mimicking the results found in Prince William Sound following the spill. In a similar fashion, Heintz *et al*. (1999) and Heintz *et al*. (2000) exposed pink salmon (*Oncorhynchus gorbuscha*) embryos to different concentrations of weathered Alaska North Slope crude oil and found very similar defects and mortality, noting that the PAHs from oil may persist in the environment for years, allowing potential population-level effects as a result of embryonic exposures. With the adverse effects of estrogen-mimicking compounds and PAHs well known for freshwater species and some preliminary evidence of similar effects for saltwater species, it could be inferred that any species of fish, freshwater and saltwater, could experience some form of disruption in biological activity or growth due to exposure to EDCs.

For this thesis project, mucus and blood plasma samples were collected from coastal pelagic fishes in the Gulf of Mexico and the Florida Straits and tested for potential endocrine disruption by detecting inappropriate vitellogenin expression with protein electrophoresis. Due to their existence in both inshore and offshore waters, coastal pelagic fishes can serve as indicator species of EDC effects, as they have the potential to be effected by large-scale environmental disasters, including acute events such as the DWH oil spill. Due to the relative ease of sample collection methods and processing, VTG detection (via presence or absence) is a low-cost, effective method of determining whether or not endocrine disruption may have taken place in any particular individual fish. Detection of VTG in males or immature females may be an indicator of

an endocrine-disrupting chemical exposure and, for this study, was used as a marker for potential contamination exposure from the DWH oil spill event. Detection of VTG in males or immature females would be an important indication of contamination, as they do not have naturally occurring detectable VTG levels in their systems. Exogenous estrogens entering the Gulf of Mexico from land-based sources would experience strong dilution once in the ocean, resulting in little to no impact on the biological systems of coastal pelagic fishes. However, any detectable VTG levels above ambient would most likely be the result of an exposure to a large input of estrogen-mimicking compounds in the open pelagic waters. During the timeframe of this experiment, any VTG detection will be inferred as a result of exposure to either spilled crude oil or dispersant chemicals revolving around the DWH oil spill event. Vitellogenin detection is an important marker of endocrine disruption because it should not be present in the blood plasma or surface mucus of male or immature female fishes. If presence is detected, it can be inferred that the natural biological processes for growth and development could be altered.

The effect of land-based estrogens that reach the ocean via rivers, lakes and streams decreases with increased dilution and distance from the effluent site (e.g., Harries *et al.*, 1997; Sumpter, 1995; and Folmar *et al.*, 1996). In the open ocean, there should be strong dilution of any river effluent and therefore the impact on marine species from land-based estrogens could be reduced compared to freshwater species, with the possible exception of shallow-water or estuarine fishes near the river output. Oil dispersants act as surfactants that lower the surface tension of the oil and allow it to form into smaller emulsion fractions, increasing surface area of the oil and thereby facilitating biodegradation (Anderson *et al.*, 1974; Ramachandran *et al.*, 2004). The theorized

purpose of oil dispersants is to break down a visible oil slick and potentially alleviate some of the risk to beaches and surface-dwelling marine animals. However, the resulting emulsification can result in an increased hazard to pelagic and bottom-dwelling marine life (Wolfe *et al*., 2001). Breaking oil down into smaller formations (as opposed to a slick) allows increased hydrocarbon exposure for fishes, which take up these hydrocarbons primarily through the gills (Ramachandran *et al*., 2004). This particulate breakdown of hydrocarbons may not only affect just fishes, but other species that live in the water column as well.

Increased exposure and bioaccumulation can also start at the phyto- and zooplankton level, both of which readily uptake PAHs, particularly in the presence of a dispersant chemical (Wolfe *et al.*, 2001). For example, uptake of PAHs from Prudhoe Bay crude oil (selected for its commonality in the North Pacific region of the United States) by a phytoplankton species (*Isochrysis galbana*, a primary producer) was enhanced by as much as 50% in the presence of chemical dispersant Corexit 9527 (Wolfe et al. 2001). The phytoplankton in this location were consumed by a rotifer (*Brachionus plicatilis*, a primary consumer), and the rotifers were in turn consumed by larval topsmelt (*Atherinops affinis*). While fishes tend to have more developed detoxification methods than phytoplankton, hydrocarbon residues were still observed in the larval topsmelt, effectively transferring PAH residues and potential adverse biological effects from the primary producers up to the larger fish species without the larger fish having to be directly exposed to PAHs (Wolfe *et al*., 2001).

The effects of endocrine disruptor chemicals on freshwater fish have been researched in several freshwater and estuarine locations on a number of species (Allen *et*

al., 1999; Moncaut *et al.*, 2003; Ramachandran *et al.*, 2006; Tarrant *et al*., 2008). To date, there have been studies performed on shallow-water coastal and estuarine species in laboratory settings, as well as preliminary studies on large pelagic fish in the Mediterranean Sea (e.g. Allen *et al*., 1999; Baklien *et al*., 1986; Fossi *et al.*, 2002; Linden, 1976). PAH uptake has been quantified for marine primary producers and fish embryonic and juvenile stages, both in the presence and absence of chemical dispersants (Wilson, 1976; Wolfe *et al*., 2001). While several freshwater species have been tested for vitellogenin in their natural habitats, saltwater species need further investigation to determine if events such as oil spills and their cleanup efforts make saltwater species equally susceptible to the negative effects of oil and dispersant exposure [e.g., lethal and sublethal toxicity (Barron *et al*, 2004; Carls *et al*, 2008), interfered reproduction (Gray and Metcalfe, 1997), reduced fecundity (Nash *et al*, 2004), developmental impairment (Wilson, 1976; Gimeno *et al*, 1997), or other unforeseen population-wide effects]. PAHs are naturally occurring in the oceans from sources such as vents and seeps. However, large inputs from man-made disasters such as oil spills put a much large EDC burden on all marine organisms and the effects on coastal pelagic species need further investigation.

Polycyclic aromatic hydrocarbons (PAHs) are organic molecules found both naturally in the environment and as an anthropogenic contaminant. They are organic molecules with two or more fused benzene rings and the stability, persistence and carcinogenic index increases with increasing number of aromatic benzene rings (Samanta *et al*., 2002). As an anthropogenic contaminant, they are often found in municipal and industrial waste effluent (Allen *et al*, 1999; Harries *et al*, 1997), crude oil and byproducts of the burning of fossil fuels (Bouloubassi and Saliot, 1991; Law and Biscaya, 1994).

While the composition of PAHs can differ, Incardona *et al*. (2009) states that crude oils are generally made up of 50 to 60 percent napthalenes, 40 to 50 percent tricyclic PAH compounds (such as fluorenes, benzothiophenes, and phenanthrenes) and one to three percent chrysenes. Fluorene is an alkylated PAH found in fossil fuels (Wang *et al*, 2003). Benzothiophene is a larger molecular weight PAH and is carcinogenic to aquatic organisms (Law and Biscaya, 1994).Phenanthrene is a lower-molecular weight tricyclic PAH with three fused benzene rings (Warren *et al*., 2012). During the weathering process, the napthalenes are the first constituents of oil to be dissolved into effluent in the highest concentrations, followed by the tricyclic (three fused benzene rings) PAHs. It is the shift to higher concentrations of tricyclic PAHs that shows overall defects and mortality to fishes occurring at much lower total dissolved PAH levels than just napthalenes (Incardona *et al*., 2009). In Anderson *et al*. (1974), hydrocarbon composition and behavior in seawater were compared for two crude oils and two refined oils, comparing their oil-in-water dispersions versus water-soluble fractions (WSF). Anderson *et al*. (1974) found the two crude oils resulted in a higher WSF compared to the refined oils. This was attributed to a higher presence of ethane-xylene, which is a light hydrocarbon that is mostly removed during the refining process. The two crude oils and the two refined oils differed significantly from one another in terms of their WSFs and the way they disperse in water, and thus differ in their relative toxicities to estuarine fish and crustaceans (Anderson *et al*., 1974). In Forns *et al.* (1977), American lobster (*Homarus americanus*) larvae were exposed to two different fractions (0.1 ppm and 1.0 ppm, respectively) of crude oil for a 14-day period while larvae were developing to their fourth stage. The larvae exposed to 0.1 ppm were actively feeding and exhibited normal

coloration (blue to blend into the pelagic zone) while the larvae exposed to 1.0 ppm exhibited almost no mobility, suppressed feeding, and exhibited red coloration (which would increase predation), which resulted in a severely decreased survival rate when reaching the second stage of development (Forns *et al.*, 1977). Regarding petrochemical spills, Baklein *et al*. (1986), found that sublethal toxicity of dispersant and dispersed oil may not have obvious effects before toxicity reaches a lethal level, which could be problematic when predicting impact of exposure on fishes that are not exhibiting any obvious symptoms. Wu *et al.* (2012) noted PAHs of different oils had a range of toxicities as a result of differing dispersabilities.

Based on the varying differences in dispersability and toxicity of different oils and oil fractions, it is important to understand the composition of the particular oil in question when it is released into the environment and how it may interact with different organisms. Reddy *et al*. (2011) collected sub-surface samples of the spilled Macondo oil (which is the oil from the oil field that the DHW rig was drilling for) directly above the seafloor leak on June 21, 2010. The composition of the oil was found to be 74% saturated hydrocarbons, 16% aromatic hydrocarbons, and 10% polar hydrocarbons (which are made up of molecules containing oxygen, nitrogen, and sulfur, many of which are resistant to evaporation or biodegradation). It was found that only about 0.01% of the methane released from the well head was free to the atmosphere, and benzene concentrations were nearly negligible near the surface waters, suggesting that both were retained in the water column (Reddy *et al.,* 2011). A significant amount of water-soluble aromatic compounds and most of the hydrocarbons released at the seafloor well head were retained in the water column and exhibited a greatly higher residence time due to

lack of opportunity to release into the atmosphere, suggesting vastly different residence times from oil spilled at the sea surface (Reddy *et al*., 2011). Reddy *et al*. (2011) also noted that the polar hydrocarbons (10% of the spilled oil) are often overlooked during field analysis studies examining the fate of the spilled Macondo oil, which leaves about 0.41 million barrels to ignored. Given the increased residence times of sub-surface spilled oil (Aitken *et al*, 2004), the circumstances of the DWH oil spill could create the opportunity for pelagic and bottom-dwelling fishes to interact with the spilled oil for a longer time frame (Brandvik *et al*, 2013; Gong *et al*, 2014). Camilli *et al*. (2010) found substantial rates of hydrocarbon loss to the atmosphere above 30 m depth with a peak in hydrocarbon abundance between 1000 m and 1300 m depth, where a subsurface plume from the leaking DWH wellhead was found and persisted for months after the initial wellhead leak. Coastal pelagic fishes reside in the surface waters and down to depths around 1000 m, where the hydrocarbon concentrations were found to be at their highest. The lack of dissolution and biodegradation at depth results in an underwater oil plume lasting longer than surface spills and creates the potential for much larger areas of water to be effected, especially given the nature of water flow and underwater currents that can transport the plume to new locations (Camilli *et al*., 2010).

While the window of exposure time may to be relatively small for any oil spill event, it is in the larval stages that PAHs may have the most impact. For inshore areas, oil particulates and dissolved constituents can remain in the sediments for long periods of time, creating the potential to influence a large number of species and generations (Bue *et al*., 1998). With a high concentration of estrogen mimics such as PAHs in the surrounding environment, fish will readily absorb the PAHs and then exhibit

physiological responses in the body that mimic conditions of circulating endogenous estrogens, causing disruptions in growth and reproduction (Sumpter and Jobling, 1995). The hydrophobic and lipophilic nature of dissolved PAHs allows them to readily cross tissue membranes, and a fish will take up dissolved hydrocarbons until steady state equilibrium is reached between the fish and water (Ramachandran *et al.*, 2006). It is also noted that the gills are a highly sensitive point of entry for hydrocarbons into the bloodstream due to their thin epithelium, high amounts of lipids, and direct contact with contaminated water (Ramachandran *et al.*, 2006). Once in the bloodstream, PAHs have the ability to travel to the vital organs such as the heart, liver, or gonads where they can bind with estrogen or androgen receptor sites (normally only occupied by natural endogenous estrogens and androgens) and trigger hormone-mediated physiological processes such as development and reproduction (Tyler *et al*., 1998). When the endocrine system is triggered at unnatural times, physiological changes and development can be greatly altered. During the early developing stages of larvae, a high concentration of estrogens has been shown to alter gonadal differentiation and could induce the formation of a testis-ovary in male larval fish (Gray and Metcalfe, 1997). In similar studies, Harries *et al.* (1997) and Cheek *et al.* (2001) also found that estrogen exposure in early life stages can cause intersexuality and feminization of male fish, including inhibition of testicular growth, impairing reproduction and decreasing hatchling success. Billiard *et al.* (1999) exposed larval zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) to dissolved retene (a PAH) and found detrimental effects such as reduced growth, fin rot, craniofacial malformations, yolk sac edema, and subcutaneous hemorrhaging, all resulting in mortality before reaching the fry stage. In Incardona *et al*. (2009), it was

shown that the weathered components of crude oil cause most damage to the developing hearts of Pacific herring (*Clupea pallasi*), leading to cardiac arrhythmia. Similarly, fertilized zebrafish embryos exposed at hatching to weathered crude oil experienced bradycardia and arrhythmia as soon as a regular heartbeat was observed (five days post-fertilization) and embryos exposed seven to eight days post-fertilization only showed irregular arrhythmia as opposed to a true developmental defect (Incardona *et al*., 2009). These differences in effects based on life stage show how PAH exposures and toxicity varies with differing developmental stages. This suggests that as fish develop and mature, they possess more ability to either move away from the contamination or have developed a more matured system of detoxification and excretion. During the Deepwater Horizon spill, Yvanka de Soysa *et al*. (2012) collected some of the spilled oil and assessed the effects of its water-accumulated fractions (WAFs) on zebrafish during embryogenesis and larval development. Similar to other studies, craniofacial deformations, swimming impairment, and cardiac and circulatory dysfunction (including cardiac edema and yolk sac edema) were observed. However, in addition, many other morphological deformations were observed. These deformations include cyst formation, dorsal tail curvature, reduced overall size of brain and eyes, caudal cysts and brain hemorrhaging (Yvanka de Soysa *et al*., 2012). While PAH exposure during larval stages may not ultimately lead to mortality, there are certainly a host of problems that can effect development, maturity, reproduction and recruitment success, even in later life stages. Khan (2013) found that reproduction of Atlantic cod (*Gadus morhua*) was altered by way of delayed spawning, spermiation, and gonadal growth. Reduced size of oocytes and spermatocytes as a result of a disruption in release of reproductive hormones following

PAH exposure was also noted, even after the cod had undergone depuration after a short exposure time of only 4 days (Khan 2013). Similarly, Moncaut *et al.* (2003) suggest that because spermatogenesis is highly regulated by hormones, exogenous estrogens may induce the renewal of germ cells at an inappropriate time, debilitating normal spermatogenesis. For sexually mature female fishes, reproduction is signaled by environmental cues such as temperature and photoperiod and regulated by endogenous hormones. Under the correct environmental conditions, these hormones stimulate the production of estradiol, a hormone secreted to trigger the synthesis of vitellogenin in the liver (Nicolas, 1999).

Vitellogenin is an egg yolk precursor protein produced by sexually mature female fishes during oogenesis. It is taken up by growing oocytes and stored as yolk to provide nutrients to growing embryos (Tyler *et al.*, 1998). Male genomes carry the vitellogenin (VTG) gene, but it is normally silent due to the low level of circulating estrogens (Jobling *et al*, 1996). However, in the presence of elevated exogenous estrogens, VTG expression can be induced in males and immature females (Moncaut *et al.*, 2003). Since males and immature females lack a deposition tissue for vitellogenin, accumulation occurs in the blood plasma and surface mucus (Hemmer *et al*., 2002). Detection of vitellogenin in the surface mucus or blood plasma of males or immature females is often used as a biomarker for endocrine disruption or to differentiate between the sexes of monomorphic fish (Kohn *et al*., 2013; Denslow *et al.*, 1999; Cheek *et al*., 2001).

Oil slicks and dispersant application in the open ocean both occur directly at the surface where larval fishes would be in direct contact with high concentrations of PAHs and estrogen-mimicking surfactants. The surface waters in the Gulf of Mexico are known

to be an important breeding ground for many large pelagic species such as bluefin tuna, whose Atlantic populations are already threatened (ICCAT 2010; Fromentin and Powers, 2005). Compromising the reproductive success and recruitment of such a species would have critical consequences for their population.

The use of chemical dispersants on oil slicks has been practiced since the 1970s. While they reduce the potential for oiling shorelines and surface-dwellers such as mammals and seabirds, chemical dispersants may increase the risk of toxicity to larval and pelagic fishes and crustaceans. An early study (Linden, 1976) outlining the effects of oil and dispersant chemical mixtures on larval Baltic herring (*Clupea harengus*) showed disastrous effects on the developing embryos. As a result of exposure to oil and dispersant mixtures, the larvae exhibited dorsal spine curvature, altered heartbeats, decreases in average body length and a swollen anterior portion of the yolk sac. Linden (1976) noted a several hundred-fold increase in toxicity of oil and dispersant mixtures versus crude oil alone. In a similar study on early dispersant use, Wilson (1976) found serious developmental impairment on three species of marine fish embryos: herring, plaice (*Pleuronected platessa*), and sole (*Solea solea*). Wilson (1976) noted that many of the embryos exposed to dispersant emulsions prior to hatching exhibited no abnormalities until post-hatch, although some did die. Upon hatching, embryos exhibited abnormalities in development such as cell cleavage, abnormal optic cup and fissure development, stunted overall larvae length, abnormal heart rates and spinal axis deformations that prevented swimming and feeding, all resulting in embryo mortality (Wilson, 1976). While early formulations of dispersants proved to be extremely toxic, newer "less toxic" formulations were implemented following the Torrey Canyon oil spill of 1967 (Wilson,

1977). However, regardless of formulation, chemical dispersants still prove to be toxic to marine life, particularly at sensitive stages of development. To further exacerbate the problem, Barron *et al.* (2003) found that the toxicity of PAHs in crude oil and oil - dispersant mixtures was greatly enhanced in the presence of natural UV light, as opposed to controlled UVA lighting in most laboratory-conditioned experiments. When exposing Pacific herring (*Clupea pallasi*) embryos and larvae to both oil-only and chemically dispersed oil mixtures, the chemically dispersed oil mixtures were significantly more toxic than oil-only treatments when exposed to natural sunlight for only ~2.5 hours per day for two days(Barron *et al.*, 2003). In addition, Barron *et al*. (2003) also found that the oil-only conditions became increasingly more toxic with increased UV exposure times. Given that most oceanic oil spills and subsequent cleanup efforts (including the application of dispersant chemicals) occur in natural UV conditions with upwards of 14 hours of sunlight exposure per day, understanding the element of photo-enhancement on toxicity may need further investigation.

In contrast, Judson *et al*. (2010) investigated the toxicity of eight commercially available chemical dispersants using in-vitro assays on single-celled organisms (including Corexit 9500; Nalco Inc., Sugarland, TX).. The chemical dispersants were considered estrogenically toxic to the cells if there was interaction between the dispersant and either the estrogen or androgen binding sites. All eight dispersants displayed no biologically significant estrogenic activity, but the authors noted that this may not be the case when considering the use of these dispersants around fish (Judson *et al*., 2010). Additionally, Judson *et al*. (2010) noted that a limitation of their study was the existence of other mechanisms of endocrine disruption that were not tested. Given that this study was based

solely on *in vitro* testing of single cells, as opposed to natural conditions in the open ocean on whole organisms, it cannot be inferred that chemical dispersants and oil and dispersant emulsions will not have some type of biological impact on larger organisms.

The Gulf of Mexico is home to a multitude of oceanic species from fish to corals, bivalves, sharks, mammals and turtles. It is also an important spawning area for many of the commercially fished pelagic species that a variety of fisheries depend on. The Gulf of Mexico is one of three major spawning grounds for Atlantic bluefin tuna, and spawning generally takes place around the month of June (Fromentin and Powers, 2005). Schwenke and Buckel (2008) note the highest percentage of ripened dolphinfish (*C. hippurus*) gonads during the late spring and early summer, with a peak in gonadal weight in the month of May, indicating a late spring to early summer spawning season. Yellowfin tuna (*T. albacores*) were found to spawn in the Gulf of Mexico between the months of May to September (Arocha *et al*., 2001). Swordfish (*X. gladius*) also exhibit a peak in spawning activity in the Gulf of Mexico from April to July (Taylor and Murphy 1992). Little tunny (*Euthynnus alletteratus*) spawn in June from the Straits of Florida and skipjack tuna (*Katsuwonus pelamis*) spawn from April to August in the Gulf of Mexico, (Klawe 1960). The spawning time frames in the Gulf of Mexico for all of these large, commercially important pelagic fishes coincided with the DWH oil spill and subsequent cleanup efforts and dispersant application. The wellhead continuously leaked oil from April 20 to September 19, 2010, i.e., throughout the entire summer and spawning season, making the likelihood of oil interaction with spawned eggs and pelagic larval stages of all of these fishes likely.

The majority of commercially important fish species, such as Atlantic bluefin tuna and dolphinfish (De Metrio *et al*., 2010; Wells and Rooker, 2009), have a planktonic egg stage followed by a pelagic larval stage where they feed on surface-dwelling zooplankton. Their distribution is restricted to the surface waters through early life stages and this puts larval fishes of a large number of species at risk during an oil spill. According to a tagging study by Weng *et al*. (2009), Atlantic yellowfin tuna in the Gulf of Mexico spend the majority of time in the surface and mixed layers. The common dolphinfish at all stages of life (larvae, juveniles and adults) are found abundantly in the Gulf of Mexico and the Florida Straits, particularly in the surface waters from 0 to 36 m (Gibbs and Collette, 1959; Palko *et al*., 1982). While the Gulf of Mexico is not a breeding ground for Atlantic bigeye tuna (*Thunnus obesus*), adults can be found in surface waters to temperatures as low as 29 ºC (Alvarado Bremer *et al*., 1998). With such a rich abundance of many species in the Gulf of Mexico during the summer months, this is a particularly sensitive time of year. Disasters such as the DWH oil spill could potentially impact many different species at any life stage. For adult pelagic fishes, one way to test for endocrine disruption (which is a potential side effect of interaction with PAHs from oil) is to test blood plasma and surface mucus for the presence of vitellogenin, which is naturally only found in adult female fishes.

There are currently two commonly used methods for detecting vitellogenin, ELISA and Western blot. In ELISA monoclonal antibody detection, or "Enzyme-Linked ImmunoSorbent Assay" (ELISA), a monoclonal antibody specific for a target protein is applied to a heterogeneous biological sample. ELISA method can quantify proteins by using calibration curves using known concentrations of the target protein. The second

method is Western blot and is used to detect proteins of a specific charge and molecular weight. The antibody binds to the protein and transfers it to a nitrocellulose membrane where is can be detected. For example, Zhong *et al*. (2014) used Western blot method to detect vitellogenin in skin and eye tissues of male zebrafish, noting that they could be used as alternatives for plasma and liver tissues. Meucci and Arukwe (2005) used a combination of ELISA and Western blot methods to detect vitellogenin in the surface mucus of Atlantic salmon (*Salmo salar*). While ELISA and western blot methods have previously been used on large pelagic fishes in the Mediterranean Sea (Fossi *et al*., 2002), Van Veld *et al*. (2005) found that phosphoprotein assay detection is at least as sensitive as the ELISA method and works better when comparing VTG measurements across multiple species. This phosphoprotein assay works universally for multiple species because VTG has a high molecular weight which allows it to be easily separated from other phosphoproteins via electrophoresis and it has a high phosphorylation at the amino acid serine, allowing it to be detected using a commercially available dye (Van Veld *et al*., 2005). For this project, the phosphoprotein assay method was used to detect and quantify VTG levels across all sampled coastal pelagic fishes. A purified rainbow trout (*Oncorhynchus mykiss*) VTG standard was compared to all wild-caught blood plasma and mucus samples through gel electrophoresis. This allowed side-by-side comparison across all sampled species. The majority of the species sampled were coastal pelagic species that had the potential to interact with the spilled oil and/or the dispersant chemicals used to break up the slick.

The impact on surface-dwelling marine species following the Deepwater Horizon oil spill event was clear. The oiled or dead seabirds, marine mammals, and sea turtles

were clear signs of oil spill damage. However, the damage to sub-surface pelagic and bottom-dwelling marine fishes is far less known. Without being able to visualize the biological impacts on these less-visible species, it is hard to evaluate the ecological impacts of the spilled oil and dispersant chemicals. Kujawinski *et al*. (2011) attempted to assess the fate of the dispersant applied at the seafloor wellhead during application. Based on calculations of dispersant and oil total volumes for the duration of the spill, there was a dispersant-to-oil ratio of 0.05% applied at the well head and it could not be determined if the dispersant application was successful at reducing oil droplet size or increasing oil degradation, as it should at the surface (Kujawinkski *et al*., 2011). For this reason, it was necessary to further investigate the extent of ecological damage impacting the marine fishes in the Gulf of Mexico.

The objective of this study was to detect contamination by endocrine disrupting chemicals in wild caught coastal pelagic fishes through the detection of vitellogenesis. Spilled crude oil and dispersants have been shown to induce vitellogenin expression in males and immature females that would not naturally do so. Mucus and blood plasma were collected from males and immature females of as many representative species as possible and compared against experimentally injected fish as well as reproductively mature females that naturally contain vitellogenin. Fish samples were obtained from commercial and recreational fishing events throughout the eastern Gulf of Mexico and around southeast Florida. This sampling effort represents species from a majority of the possible contamination areas in the months following the Deepwater Horizon oil spill. Given the unique ecotone presence by coastal pelagic fishes, they provide a vital trophic link between the inshore and offshore habitats and thus serve as indicator species for

EDC presence. Collection of blood and mucus samples of males and immature females of these fishes were tested for presence and prevalence of VTG and compared to experimentally induced VTG controls.

Methodology

Coastal pelagic fishes (e.g., yellowfin tuna, blackfin tuna (*Thunnus atlanticus*), dolphinfishes) and pelagic fishes (e.g., swordfish) were sampled via mucus and/or blood plasma collection through offshore fishing trips on board commercial pelagic longline, swordfish buoy gear, and tuna greenstick fishing vessels. Research and recreational fishing vessels were also opportunistically used to collect samples in the Gulf of Mexico, along the coast of Broward and Miami-Dade counties, and other areas throughout the Florida Straits. Onshore sampling took place at large recreational fishing tournaments throughout Southeast Florida and the Florida Keys.

Mucus Collection

For each sampled fish, mucus was scraped from a 4 cm^2 (or larger, depending on available amount of mucus) area of skin using a clean metal scraper. If the fish was freshly caught, such as on a pelagic longline vessel, mucus was taken from the skin near the dorsal fin, as long as there had been no possible contamination from touching other fish or a surface where another fish may have been placed. If the fish was sampled at a tournament and there was risk of cross-contamination such as in a cooler or fish hold, a more protected area such as under the lip of the operculum or just inside the mouth was scraped.

The mucus was transferred to aprotinin-treated 1.5 ml microcentrifuge tubes (Fisher Scientific) containing 250 µl of phosphate-buffered saline and placed on ice or in a liquid nitrogen dewar. Aprotinin is a protease inhibitor, which keeps proteins in the mucus and plasma from being degraded by the naturally occurring or contaminating

proteases. Samples were labeled, documented and placed in a -80 ºC freezer until transfer to the processing laboratory at Coastal Carolina University in Conway, SC.

Blood Plasma Collection

Blood plasma samples were collected using 1.5 inch, 21-gauge aprotinin-treated syringes inserted into the heart or caudal vein, depending on the size of the individual fish being sampled. The blood was then injected into 10 ml glass evacuated plasma tubes containing freeze-dried sodium heparin anticoagulant and placed on ice until it could be centrifuged. Centrifuging the blood allowed separation of the cellular fraction from the plasma. The plasma was then collected using a 1.5 inch, 21-gauge aprotinin-treated syringe, deposited into an aprotonin-treated 1.5 ml microcentrifuge tube and stored at -80 ºC until transfer to Coastal Carolina University. For some of the commercial fishing trips longer than one day, a portable centrifuge was taken aboard the vessels to allow for same-day plasma separation to ensure quality of the sample. For longer trips where a portable centrifuge was not feasible, mucus collection was the main method for protein collection.

Fishing Tournament and Day Charter Boat Sampling

At each fishing tournament, a sampling station was set up at or near the dock where anglers could voluntarily bring their catch to have samples taken. Once a sample was collected, it was placed in a cooler with ice until return to the laboratory in Dania Beach, FL. Once back in Dania Beach, samples were labeled, documented, and placed in a -80 ºC freezer until transfer to Coastal Carolina University. During both fishing

tournaments and for day-charter vessels, the main fishing method used was rod-and-reel fishing where each individual angler had a fishing rod with reel.

Commercial Fishing Operation Sampling

While aboard commercial fishing vessels for other fisheries-related projects, samples were collected opportunistically from all coastal pelagic species caught during fishing operations. The main vessels utilized for fishery projects and subsequent data collections were the F/V *Sun Dancer* and F/V *Miss Rita*, both from Madeira Beach, FL. During fishing operations, samples were collected immediately after the fish were brought on board. Once collected, mucus and blood samples were set on ice in an on-deck cooler. At the conclusion of fishing operations each day, mucus samples were labeled and transferred to a -20˚C freezer. Blood samples would remain in the evacuated plasma tube until they could be spun at the end of the day. The plasma was then collected, labeled, and transferred to a freezer. Samples remained in the freezer until the conclusion of the trip (average 14 days). Samples were then transferred via cooler from Madeira Beach, FL back to the laboratory in Dania Beach, FL. Once back in Dania Beach, samples were labeled, documented, and placed in a -80 ºC freezer.

There were two main commercial fishing methods utilized by the vessels included in this project. The first gear type was greenstick fishing gear. The second gear type was commercial longline fishing gear. A third gear type used less frequently was commercial swordfish buoy gear. Examples and explanations for all gear types can be found at http://www.seagrantfish.lsu.edu/management/longlines.htm.

Experimental Controls

Experimental controls were created using crevalle jacks (*Caranx hippos*) and lookdowns (*Selene vomer*), which were the most readily-available and phylogenetically similar coastal pelagic species that could be caught, injected and kept alive. These fish were caught using rod and reel at the Nova Southeastern University's Oceanographic Center. Upon being caught, each jack and lookdown was weighed and injected with estradiol-17β (at 10 µg/g body weight) into the peritoneal cavity. After injection, each fish was placed in one of two 1400-gallon recirculating salt water tanks. The fish remained in the tanks for a minimum of 72 hours to allow vitellogenesis to occur. At the completion of the 72 hours, each fish was removed from the tanks and humanely euthanized via pithing. Then, the blood plasma and mucus was collected, labeled and stored until processing.

Sample Processing

All collected samples were labeled, sorted by species and sample type (mucus or blood plasma) and remained in -80 ºC storage until transfer (via either overnight shipping in a cooler with ice packs or driven in a vehicle and kept on dry ice in a cooler) to the processing laboratory at Coastal Carolina University in Conway, SC. Once at the laboratory, each sample was thawed once and aliquoted into multiple microcentrifuge tubes. As many aliquots as possible of each sample were made (based on total volume of original sample). Each aliquot was used once, either in protein analysis (using the Qubit® 2.0 fluorometer to assess protein concentration in the sample) or gel electrophoresis (to

separate the proteins in the sample). Typically, protein analysis and gel electrophoresis steps were done on two different days so a full sample analysis used two aliquots. Aliquoting was done to preserve the sample integrity by preventing multiple thawing and freezing cycles of the original sample.

Protein Assays

Sample assays were processed using a Qubit® 2.0 Fluorometer, Qubit® reagent, Qubit® protein buffer and three running standards needed to calibrate the fluorometer (Qubit® supplies, Invitrogen). When ready to process, samples were removed from the freezer and allowed to thaw completely (but remain chilled, so to not reach room temperature). Before using the reagent, air bubbles were centrifuged out to ensure accurate volumetric measurements. A working solution was created using the Qubit® reagent and protein buffer in a 1 µL reagent:199 µL buffer ratio and then spun on a vortex mixer for 3 seconds to ensure even mixing. All samples and standards were prepared in appropriately labeled Qubit® assay tubes (Invitrogen Q32856 tubes). Each of the three running standards was separately prepared with 190 µL of the working solution and 10 µL of standard and then spun on the vortex mixer for two to three seconds. Each sample tube was prepared with 190 µL working solution and 10 µL of sample solution (ensuring sample was free of particulates such as flesh or scales) and spun on the vortex mixer for two to three seconds. All sample and standard tubes incubated in the dark at room temperature for 15 minutes and then read by the Qubit® Fluorometer to obtain protein concentrations for each sample.

Gel Electrophoresis

The Qubit® fluorometer protein concentrations given in the previous steps provided a volume of sample needed to provide a protein mass between one and five µg in each prepared sample. The protein mass of 1-5 µg of sample was the amount necessary to be utilized in the electrophoresis procedures (Van Veld *et al*., 2005). For this experiment, a protein mass of 5 µg of each sample was used.

A running buffer solution was prepared using Laemmli sample running buffer (Bio-Rad Laboratories, Inc.; Hercules, CA, USA) and β- mercaptoethanol at a concentration of 50 µL of β- mercaptoethanol to 950 µL of Laemmli sample buffer. The sample buffer and sample solutions required a 1:1 ratio of sample:buffer/β-mercaptoethanol solution. Once each tube was filled with running buffer and sample solution, they were vortex mixed and heated on a heat block for five minutes between 90 and 100°C. Purified lyophilized rainbow trout (*Oncorhynchus mykiss*) vitellogenin (Cayman Chemical) was used as a vitellogenic standard. It required reconstitution to 0.001 µg/µL in phosphate-buffered saline.

A Mini-PROTEAN® Tetra Cell 4-gel electrophoresis system with 7.5% Mini-PROTEAN TGX™ Precast Gels with 12 (20 µL) wells were used for the electrophoresis processing of the samples. Precision Plus Protein™ Dual-Color Standard (Bio-Rad®) was used as a molecular weight ladder. Gels were loaded into the module, running buffer was poured into the module, and the standards and samples were loaded into the gels. The gels were allowed to run at 200 volts for approximately 20 min, or until the dye front almost reached the bottom of the gel.

While the gels were running, the fixation liquid was prepared for the staining procedures. Pro-Q Diamond® phosphoprotein gel staining solution (Life Technologies®, Grand Island, NY) and Coomassie Brilliant Blue R-250 staining and destaining solutions (Bio-Rad Laboratories, Inc.) were used to identify phosphoproteins in the samples. For gels stained with the Coomassie Brilliant Blue® stain, no fixation liquid was required. When the dye front on the gels reached the bottom of the gel, the electricity to the module was turned off and the gel cassettes were carefully removed. Each gel was placed in a plastic container, just larger than the size of the gel for fixation and staining.

Gel Staining

Pro-Q Diamond® Gel Staining: The standard staining protocol for tris-glycine gels was followed (Life Technologies, Fisher Scientific, Grand Island, NY, USA). The gels were agitated in fixation liquid for 30 min in the dark to prevent photobleaching. All gel agitation was done using a GeneMate® Low Speed Orbital Shaker (BioExpress®). At the end of the 30 min, the fixation liquid was poured off and 100 mL of fresh fixation liquid was added and the gels were agitated for another 30 min in the dark. The gels were then washed by agitating three times with 100 mL of nanopure water for 10 min, for a total time of 30 min. After washing steps, the gels were stained using 60 mL of Pro-Q® Diamond staining solution. They were agitated in the staining solution for 90 minutes. After staining, the gels were destained in 100 mL of destaining solution, agitated for 30 min. Destaining was repeated twice more, for a total of 90 min. The gels were then washed with 100 mL of nanopure water for 5 min, repeating twice.

Coomassie Gel Staining: The standard protocol for Coomassie Brilliant Blue® staining was followed (Bio-Rad, Hercules, CA, USA). Each gel was placed in 100 mL of Coomassie Brilliant Blue® staining solution. The gels were agitated with the stain in the dark for 60 min. The gel stain was poured off and 100 mL of Coomassie Brilliant Blue® destaining solution was poured onto the gels. Four Kimwipes® were twisted together into two pairs and laid along the outside edges of the container, careful to not touch the gel. The gels and Kimwipes® were agitated on the orbital shaker in the dark for 60 min. The Kimwipes® were replaced with fresh ones and allowed to shake for an additional 30 min or until the desired level of destaining was reached.

Gel Illumination and Photographing

Following staining, fluorescent images of the gels stained with Pro-Q Diamond® staining solution were captured using a Kodak® Gel Logic 100 imaging system (Kodak®, 2000). The images of gels stained using the Coomassie Brilliant Blue® staining techniques were photographed using a Panasonic® Lumix TS3 digital camera.

Data Analysis

All wild-caught and control samples were electrophoresed using a Mini-PROTEAN® Tetra Cell 4-gel electrophoresis system to separate the proteins in the blood plasma and mucus samples, which were then stained using Pro-Q Diamond® phosphoprotein gel staining solution (Life Technologies®, Grand Island, NY) and Coomassie Brilliant Blue R-250 staining and destaining solutions (Bio-Rad Laboratories, Inc.). The staining processes allowed detection of phosphoproteins in the wild-caught and

control samples and allowed comparison of the samples to the purified rainbow trout (*O. mykiss*) VTG standard.

Results

A total of 228 coastal pelagic fishes were sampled by collecting surface mucus and/or blood plasma from each fish. These collected samples were from 110 yellowfin tuna (mean = 113.1 cm FL; range = 69 to 169 cm FL; standard deviation = 19.16), 27 blackfin tuna (mean = 66.6 cm FL; range = 49 to 90 cm FL; standard deviation = 13.12), 25 little tunny (mean = 60 cm FL; range = 42.3 to 76.2 cm FL; standard deviation = 8.36), ten skipjack tuna (mean = 63 cm FL; range = 52 to 79 cm FL; standard deviation = 8.90), five bigeye tuna (mean = 96.8 cm FL; range = 90 to 104 cm FL; standard deviation = 6.42), one bonita (*Sarda sarda*) (64 cm FL), 25 swordfish (mean = 146.3 cm FL; range = 104.6 to 185 cm FL; standard deviation = 22.13), ten common dolphinfish (mean = 74 cm FL; range = 28.5 to 117 cm FL; standard deviation = 27.70), three wahoo (*Acanthocybium solandri*) (mean = 121.7 cm FL; range = 101 to 146 cm FL; standard deviation = 22.72), two king mackerel (*Scomberomorus cavalla*) (mean = 84.2 cm FL; range = 82.4 to 86 cm FL; standard deviation = 2.55) and one Atlantic sailfish (*Istiophorus albicans*) (203.2 cm TL). There were also two pinfish (*Lagodon rhomboides*) (mean = 23.6 cm FL; range = 22 to 25.2 cm FL; standard deviation = 2.26), two escolar (*Lepidocybium flavobrunneum*) (mean = 99.7 cm FL; range = 94.6 to 104.8 cm FL; standard deviation = 7.21), two snowy grouper (*Epinephelus niveatus*) (mean = 51.2 cm TL; range = 50.1 to 52.3 cm TL; standard deviation = 1.56), and one gulf flounder (*Paralichthys albiguttata*) (42 cm FL) (Table 1 and Figure 1).

Of the 228 fish sampled, only 25 samples (17 from blood plasma and 8 from mucus) showed clear protein banding on the gel cassettes after processing and staining (photo images of gel cassettes can be found in Appendices 1 through 3). These 25 samples

	YFT	BLK	LTA	SKJ	BET	BON	SWO	DOL	WAH	KGM	SAI	PIN	ESC	SNG	GLF
Mean (cm, TL)	113.1	66.6	60	63	96.8	64	146.3	74	121.7	84.2	203.2	23.6	99.7	51.2	42
Range (cm, TL)	69-169	46-90	42.3-76.2	52-79	90-104		104.6-185	28.5-117	101-146	82.4-86		22-25.2	94.6-104.8	50.1-52.3	
Stan. Dev	19.16	13.12	8.4	8.9	6.42		22.13	27.7	22.72	2.55		2.62	7.21	1.56	

Table 1: Mean, range and standard deviation for all species sampled. Measurements are in centimeters total fork length (cm, TL).

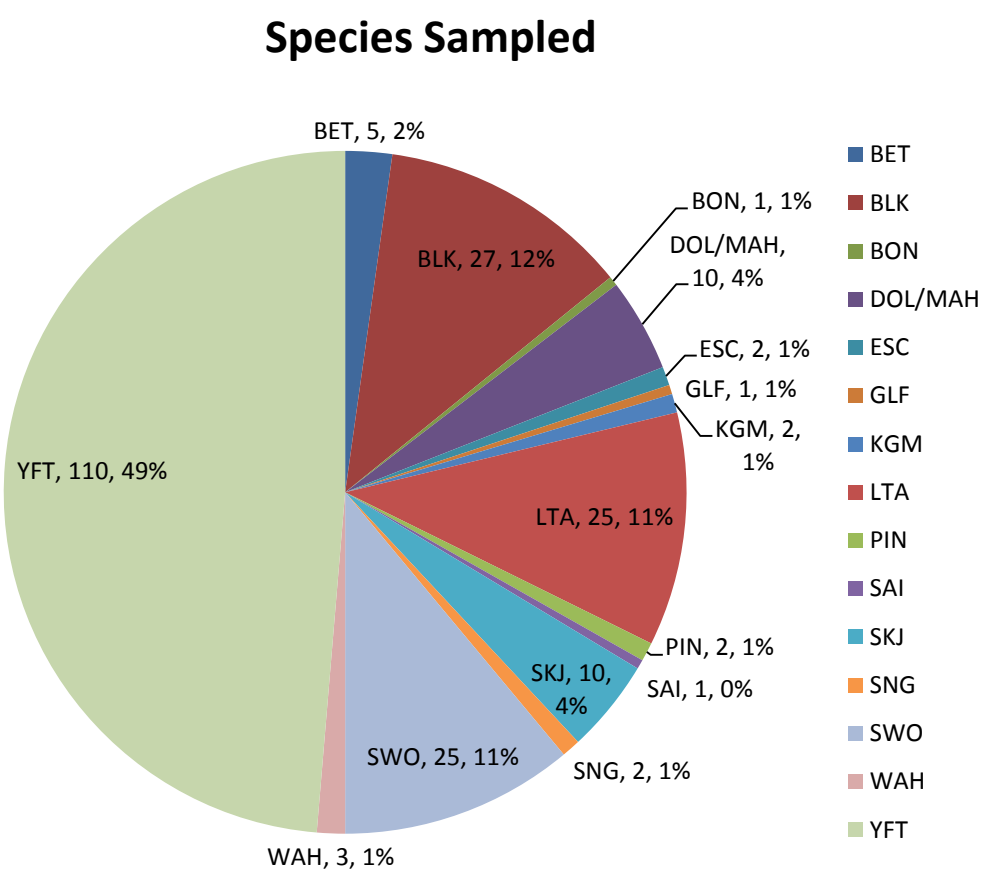

Figure 1: Species breakdown of all coastal pelagic fishes sampled, labeled as species, number sampled and species percentage of entire sample set. A total of 228 coastal pelagic fishes were sampled, the majority being yellowfin tuna. BET = bigeye tuna; BLK = blackfin tuna; BON = bonita; DOL/MAH = dolphinfish/mahi mahi; ESC = escolar; GLF = gulf flounder; KGM = king mackerel; LTA = little tunny; PIN = pinfish; SAI = sailfish; SKJ = skipjack tuna; SNG = snowy grouper; SWO = swordfish; WAH = wahoo; YFT = yellowfin tuna.

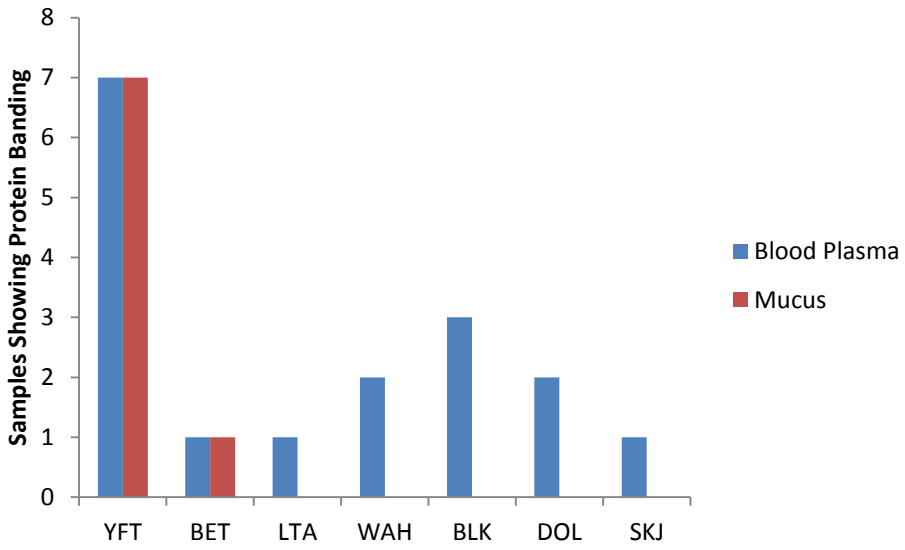

Figure 2: Number of each sample type that showed clear protein banding on gel cassettes following processing; separated by species. A total of 206 mucus samples were collected. Of those 206, 162 were collected before realizing the phosphate-buffered saline preservative solution was concentrated (25X). Of the 44 properly-preserved mucus samples, only 8 resulted in a viable sample that provided clear banding on the gel cassettes following processing. BET = bigeye tuna; BLK = blackfin tuna; BON = bonita; DOL/MAH = dolphinfish/mahi mahi; ESC = escolar; GLF = gulf flounder; KGM = king mackerel; LTA = little tunny; PIN = pinfish; SAI = sailfish; SKJ = skipjack tuna; SNG = snowy grouper; SWO = swordfish; WAH = wahoo; YFT = yellowfin tuna.

consisted of seven yellowfin tuna blood plasma samples, seven yellowfin tuna mucus samples, one bigeye tuna blood plasma sample, one bigeye tuna mucus sample, one little tunny blood plasma sample, two wahoo blood plasma samples, three blackfin tuna blood plasma samples, 2 common dolphinfish blood plasma samples and 1 skipjack tuna blood plasma sample (Figure 2). These 25 fish were caught using the fishing methods outlined in Chapter 2: Methodology (2.1 Experimental Set-Up). Twenty-one of these fish were caught using commercial greenstick gear, two were caught using rod-and-reel angling on day charter vessels and two were caught using pelagic longline gear utilized by the research vessel R/V *Weatherbird II* (Florida Institute of Oceanography; St. Petersburg, FL) (Figure 3). These samples were collected from the Gulf of Mexico.

For this experiment, only 11% of the total collected sample set resulted in viable samples (25 out of 228 total fish sampled) for analysis. Of the 25 viable samples, zero samples showed any fluorescent banding in the 150-kDa (kilodalton) molecular weight protein range, which is indicative of a phosphoprotein of the expected molecular weight of vitellogenin (Figure 4). The catch locations of these 25 viable samples are shown in Figure 5. These catch locations coincide with known contamination areas outlined by NOAA (National Oceanic and Atmospheric Administration) following the DWH spill and cleanup efforts (Figure 6).

A total of 60 control-injected fish (39 crevalle jacks and 21 lookdowns) were used as experimental vitellogenic controls. Of the 60 control-injected fish, only two (3.3 %) crevalle jacks were viable. The two viable controls did exhibit protein banding in the 150-kDa range, indicating a presence of vitellogenin.

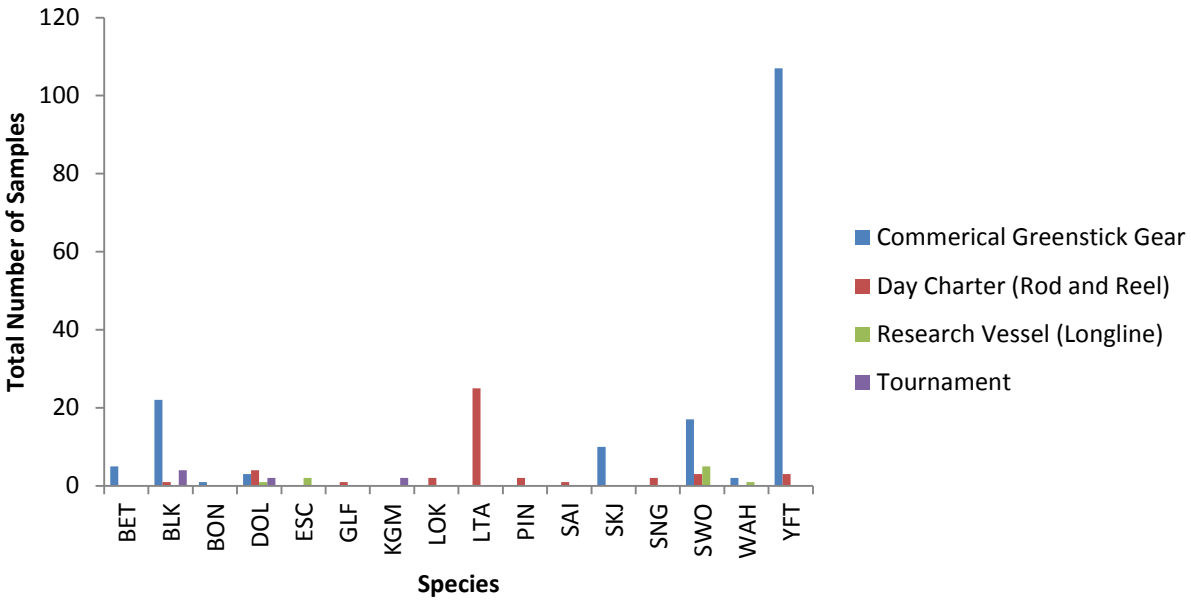

Figure 3: Collection method used for each sample collected for this project. Collection methods utilized: commercial greenstick fishing gear, rod-and-reel angling on day charter vessels, pelagic longline fishing on a research vessel (R/V *Weatherbird II*, St. Petersburg, FL), and tournament sampling. All sampling methods are outlined in Chapter 2, Methodology. BET = bigeye tuna; BLK = blackfin tuna; BON = bonita; DOL/MAH = dolphinfish/mahi mahi; ESC = escolar; GLF = gulf flounder; KGM = king mackerel; LTA = little tunny; PIN = pinfish; SAI = sailfish; SKJ = skipjack tuna; SNG = snowy grouper; SWO = swordfish; WAH = wahoo; YFT = yellowfin tuna.

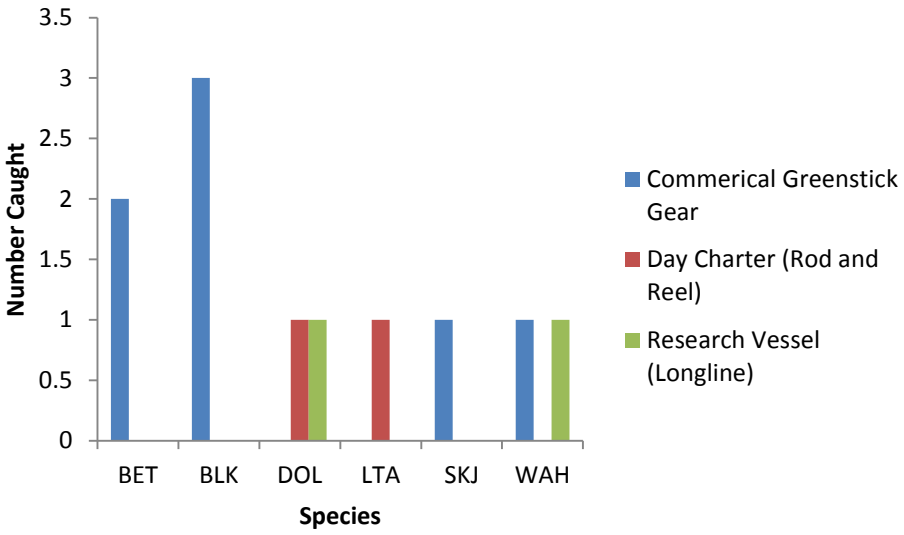

Figure 4: Number of each species caught that showed clear protein banding on gel cassettes following processing; separated by species and catch vessel type. The three vessel catch methods that yielded viable samples were commercial greenstick tuna fishing gear, rod and reel hand fishing on day charter vessels and traditional tuna trolling gear on a research vessel. BET = bigeye tuna; BLK = blackfin tuna; DOL = dolphinfish; LTA = little tunny; SKJ = skipjack tuna; WAH = wahoo.

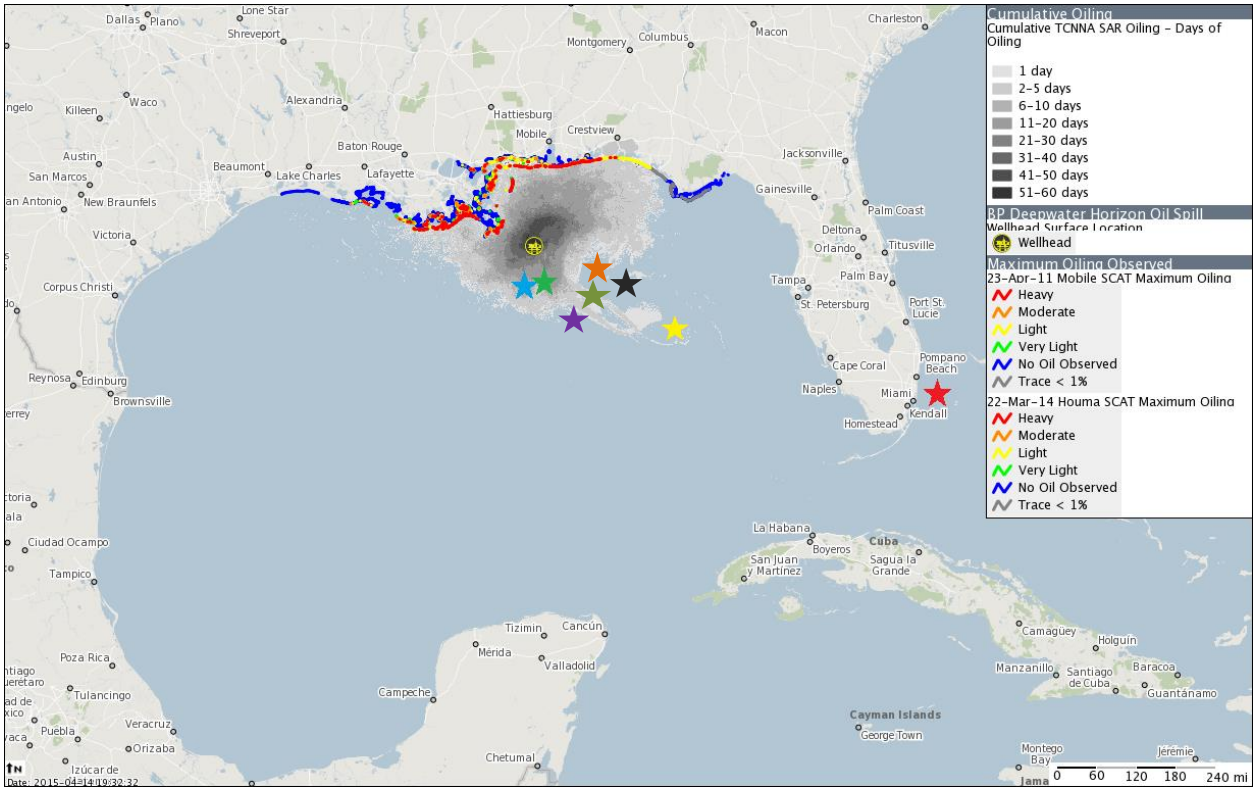

Figure 5: A map of the Gulf of Mexico showing catch locations of the 25 viable sampled fish collected for this experiment. Note: multiple fish were caught at some of the locations, accounting for multiple fish and multiple species in a single location. Image downloaded April 2015 from www.GeoPlatform.gov/gulfresponse

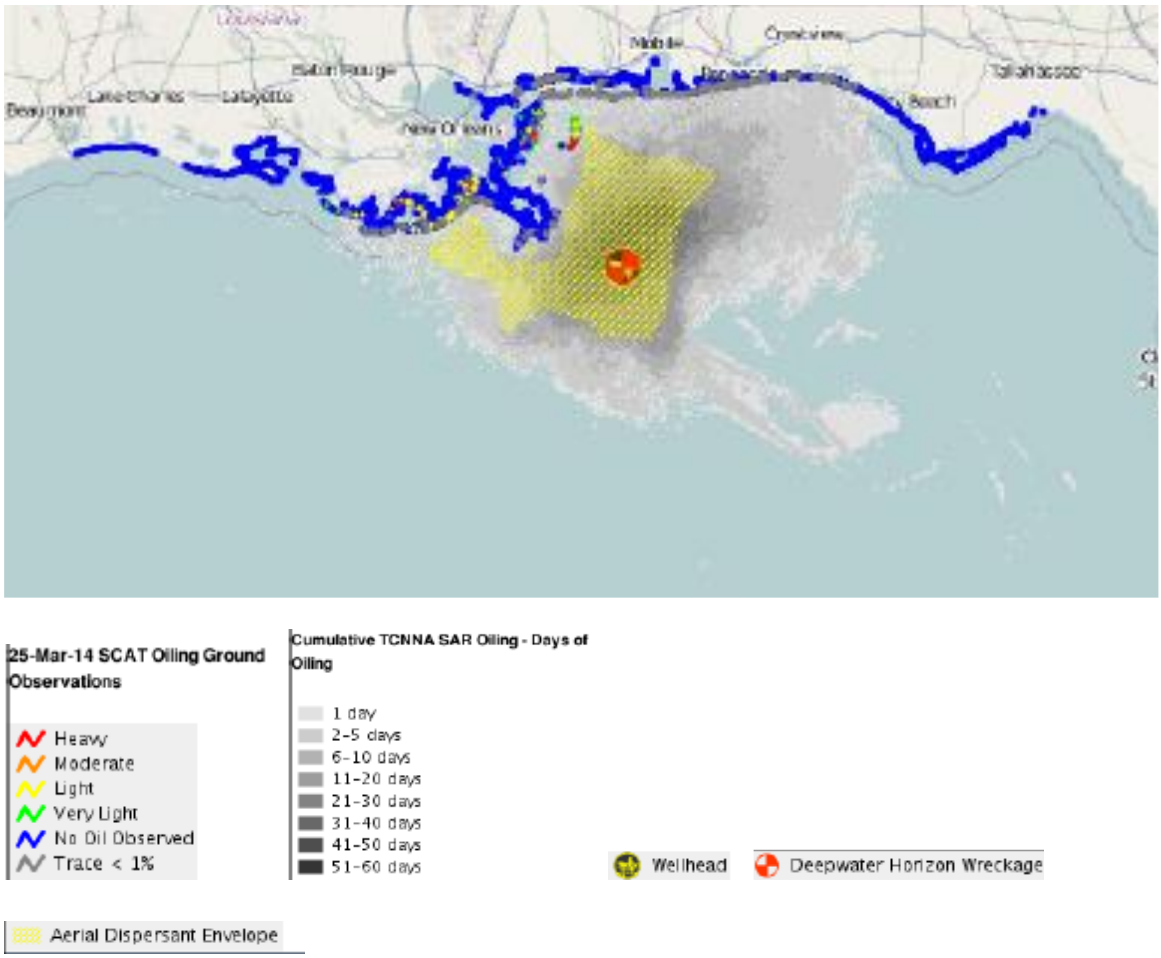

Figure 6: A map of the Gulf of Mexico showing oiled shorelines, extend of oil aerial dispersant coverage and the location of the wellhead and oil rig wreckage. Image downloaded June 2014 from www.GeoPlatform.gov/gulfresponse.

During processing in December of 2012 it was discovered that the PBS for sample storage was in concentrated form (25×), causing the proteins in the mucus and blood plasma to bind with the excess salts. This resulted in a precipitate "clump" of salt and protein that was unable to be loaded on the gels for electrophoresis. As a result, there were only two crevalle jack samples that were not either contaminated by the highly concentrated PBS or degraded due to thawing and refreezing, making them the only two working experimentally-injected controls.

Blood plasma collection protocol required the use of a portable centrifuge to separate the plasma from the red blood cells. Separation of the plasma exhibited best results when the blood was centrifuged directly after collection. The use of a portable centrifuge was used on the first commercial fishing trip on F/V *Sundancer*. The interior structure and electrical wiring of the vessel allowed daily use of the centrifuge to collect blood plasma. On subsequent commercial

trips on the F/V *Miss Rita*, there were no adequate areas to set up the centrifuge and no access to an electrical supply. For these trips, mucus was the main sample collected.

Discussion

The results of this experiment indicate that there was an absence of endocrine disrupting chemicals within the sampled coastal pelagic fish complex in the Gulf of Mexico and along the South Florida shelf. None of the 25 viable samples collected from various coastal pelagic species showed any endocrine disruption via vitellogenin detection. The lack of vitellogenin in any sample suggests that none of the collected fish experienced an increase of estrogenic activity from exposure to any of the spilled Macondo crude oil or chemical dispersants. Given the absence of endocrine disrupting chemical exposure, the effects of trophic position on endocrine disrupting chemical prevalence within the coastal pelagic species was not able to be evaluated.

The sample size for this project was significantly lessened because a large number of the samples (89%) were not able to be processed successfully, due to sample contamination, degradation or improper sample preservation. For the first 24 months of sample collection, all of the mucus samples were preserved using a concentrated (25X) phosphate-buffered saline solution. I did not realize the phosphate buffered saline was in concentrated form until processing the samples in December of 2012. The concentrated phosphate-buffered saline resulted clumping up (or "salting out") of the sample proteins after being mixed with the sample running buffer solution (Laemmli sample running buffer (Bio-Rad®) and β- mercaptoethanol). This effectively rendered all mucus samples unusable, leaving only a very small blood plasma sample set (25 usable samples out of 228 total samples). The mucus samples comprised a large majority of the total sample volume due to the ease of its collection versus blood plasma. Starting in March of 2013,

sample collection resumed on commercial fishing vessels using appropriately diluted phosphate-buffered saline.

I believe large impedance resulting in a lack of results for this experiment was the sampling time frame following the oil spill. The spill occurred from April to September of 2010. The approval time to move forth with this experiment, along with finding sampling avenues, resulted in the first sample collection occurring December of 2011, a full sixteen months after the Macondo well had been capped. While the presence of large underwater oil plumes and surface slicks still existed during times of sample collection, the likelihood of a fish experiencing contamination resulting in vitellogenin induction and thus detection by this assay were probably minimal. It has been found that vitellogenin clearance from the mucus and blood plasma of adult fish may take as little as two days following exposure, making this type of exposure detection extremely time-sensitive. Hemmer *et* al. (2002) exposed male sheepshead minnows (*Cyprinodon variegates*) to either 17 β-estradiol or *para*-nonylphenol for 16 days and then measured vitellogenin levels after cessation. It was found that the minnows exhibited a 94% decrease in vitellogenin mRNA levels 4 days post-exposure and after 8 days, vitellogenin levels were back to basal levels (Hemmer *et al*., 2002). Hemmer *et al*. (2002) also note a dose-dependent clearance rate of plasma vitellogenin levels but noticed a peak in plasma VTG levels two to four days following cessation, suggesting accumulated estrogen residues possibly stimulating VTG transcription for a short period after exposure. With such short residence times in the mucus and plasma of fishes, samples must be collected almost immediately following exposure to be able to detect any elevated levels of vitellogenin. While the possibility of large, underwater oil plumes still existed in the Gulf of Mexico in

the months following the oil spill, theorized contamination via vitellogenin detection in the mucus or blood plasma would most likely yield the best results from fish that were just exposed to the oil and dispersant chemicals almost immediately before sample collection. The first samples collected for this project were from December 2011, three months following the oil spill. The last samples were collected in May of 2013, almost two years after the oil spill. A major reason behind the extended time frame of sample collection was the improper preservation of all mucus samples collected in the first two years. Sample collection essentially had to start over in early 2013, extending the timeframe of this project much longer.

Sample collection was hampered during the summer and fall seasons due to offshore winds and storms associated with hurricane season in the Gulf of Mexico and the Florida Straits. Some of the commercial fishing trips were delayed for weeks at a time due to weather constraints.

Another constraint for this experiment regards the field collection methodology. Collection and preservation techniques were not stringent enough to provide a strong sample quality required for protein gel electrophoresis. The collection and processing methodologies followed the techniques used by Van Veld *et al*. (2005). However, sample collection and storage methods utilized during this experiment were not to the standards required to maintain optimum sample quality. The collection and preservation methods outlined in Section 2.1 detail the process of sample collecting and storage while on commercial fishing vessels. Proteins in mucus and blood plasma can be very easily degraded in a short amount of time. Storing the collected samples in a cooler with ice on the deck of the vessels was not sufficient to keep the samples cool enough prior to being

placed in the freezer. More often than not, the ice would be melted hours before the samples were able to be frozen and the temperature inside the cooler was too warm to preserve the proteins. Also, access to electricity for the portable centrifuge was infrequent and only available in the evenings. Blood plasma that had been collected throughout the day and placed in the coolers on deck was not able to be spun until up to 8 hours after being collected (if collected in the morning and not able to be spun until that night). Blood plasma separates from the platelets best when it is spun soon after being caught. This coupled with the warm cooler temperatures made blood plasma collection very difficult and typically resulted in unusable samples. To further exacerbate the problem, the F/V *Sundancer* (the vessel that yielded the highest number of collected samples) had a limited supply of generator power. Therefore, the freezer would only be run every other night in order to keep the food frozen enough to prevent spoiling. This limited freezer access to only times when it was running so blood plasma samples could only be placed in the freezer every other night. Also, the restricted generator power meant the freezer was typically warmer than normal temperatures. If a sample had been collected on one of the first days of a multi-week trip, the sample integrity was most likely not preserved and rendered it unusable. While the use of commercial fishing vessels as the main method of sample collection allowed access to species and fish sizes not typically accessible through other fishing avenues (day charters, rod-and-reel angling from shore or dock, etc.), the available sample preservation fell short due to being limited to what equipment was available on the vessel or what could be taken aboard the vessel.

Commercial fishing vessels allow for a sampling opportunity with fresh access to a variety of large coastal pelagic species. However, given the lack of sample quality from

this experiment, it must be noted that sample preservation techniques need to be extremely stringent. As found during sample collection and processing, commercial vessels may not allow for adequate sample storage after collection. Proper equipment and immediate access to a freezer are absolutely required when dealing with time and temperature-degraded samples such as proteins. At the start of this project, a nitrogen dewar was purchased in hopes that it could solve the issue of immediate freezing of samples. However, the commercial fishing trips lasted much longer than the supply of liquid nitrogen in the dewar would last so the use of the dewar became infeasible. The research vessel (R/V *Weatherbird II*) was an excellent combination of fresh fish access using commercial fishing gear and adequate processing and storage facilities immediately following sample collection (an on-board laboratory facility included a centrifuge and a -80°C freezer).

Sample collection and sample processing for this project happened in two different locations. Sample collection occurred in the Gulf of Mexico and the Florida Straits by way of commercial fishing vessels, research vessel and charter vessels. Samples were stored at the Nova Southeastern University Oceanographic Center in Dania Beach, FL. All laboratory processing procedures took place at Coastal Carolina University in Conway, SC. Samples were transported via vehicle (either FedEx overnight shipping or driving a personal vehicle) from Dania Beach, FL to Conway, SC. Given the distance between laboratory locations, sample processing only occurred on three different occasions (June 2012, November to December 2012 and January to February 2014). For future studies, it would be beneficial to do all sample processing simultaneously with collection. This would ensure that any problems (such as sample contamination or

degradation of quality) would be noticed prior to having a large sample collection and large amounts of time gone by. For this project, the infrequent bouts of sample processing did not allow for the realization of sample contamination from the concentrated phosphate-buffered saline until after a large sample set (just under 200 samples) had already been collected and thus contaminated. This wasted a large amount of time and set the timeframe of this project back by almost two years. As discussed earlier, the detection of endocrine disruption via vitellogenesis is a time-sensitive response and the longer the time frame of sample collection after the oil spill, the less likelihood of detecting contamination in the samples. Also, transfer of samples from storage in Florida to the processing facility in South Carolina more than likely resulted in either partial or complete thawing of the samples, degrading the quality. Sample collection and processing at the same facility would have greatly increased the quality of samples and the size of the sample set. It is also important to note that this was the first project of its kind for all individuals involved at the facility in Dania Beach, FL. All individuals involved in this project were all learning at each step of the process. Now we are all aware how stringent the sample and storage protocols need to be when working with proteins.

However, it can be inferred that gel electrophoresis and the phosphoprotein staining techniques used in this experiment do work for this type of protein detection, due to the presence of protein banding in the wild-caught samples and the presence of a protein band at the 150-kDa molecular weight range for the experimentally injected control fish.

For future studies, commercial fishing vessels or research vessels utilizing commercial fishing gears would be the best way to access a variety or live coastal pelagic fishes to get the best sample quality possible. Make sure there is immediate freezer access to ensure preservation of sample quality. It would be best to start sample collection immediately following a potential interaction with contaminants (i.e., immediately following the oil spill, as was the case for this study). This would ensure the highest probability of contaminant detection. Also, I would recommend sample collection and processing occur at the same location and be done consecutively. This would allow for a more immediate detection of any problems, allowing for the most accurate results when dealing with constrained time frames.

For future studies, knowing the composition of the oil and dispersant chemicals used and investigating how they simultaneously interact with the species being investigated would be critical to understanding how the fish could be affected. To date, there is mixed evidence as to whether or not dispersant chemicals increase or decrease toxicity to marine species. Linden (1976) found the emulsion of oil and dispersant to exhibit a series of sublethal and lethal effects on Baltic herring (*Clupea harengus membras*) and the toxicity of crude oil increased by several-hundred fold if the oil is in an emulsion with a dispersant. Following the Deepwater Horizon spill in the Gulf of Mexico, Rico-Martínez *et al*. (2013) investigated the collaborative toxicity of Macondo oil with Corexit 9500A®, the main dispersant used during the spill. A marine rotifer *Branchionus plicatilis* was exposed to varying ratios of Corexit 9500A®: Macondo oil in 1:10, 1:50 and 1:130 fractions. The 1:10 and 1:50 Corexit application ratios were recommended by the EPA but the 1:130 ratio (roughly 4.9 million gallons of spilled

Macondo oil to about 2 million gallons of dispersant applied) was the amount actually used in the Gulf of Mexico (Rico-Martínez *et al*., 2013). The rotifers exhibited a 52-fold increase in toxicity (through cyst formation and mortality) when exposed to all ratios of 9500A®: Macondo oil, versus exposure to either component alone Rico-Martínez *et al*., 2013). Barron (2012) conducted a summarization of major research efforts following the Deepwater Horizon spill and concluded that immunotoxicity is commonly overlooked in major environmental assessments following large-scale oil spills, as it was for this spill. Though it has been shown that oil and dispersant chemicals are toxic to all forms of marine life, a lack of research involving immontoxic responses in potentially affected organisms could be a serious shortcoming as populations may be slow to recover in the future as a result of impaired disease resistance and increased parasitism following immunotoxicity (Barron, 2012).

Conclusion

The results presented from the conclusion of this experiment suggest that no coastal pelagic species tested were contaminated by exposure to the PAHs in the spilled Macondo crude oil or the dispersant chemicals used to clean up the spill. However, with such a small viable sample size, these results are not significant enough to determine the extent of endocrine disruption from the oil spill or theorize how many fishes were exposed, if any at all. Also, a lack of vitellogenin expression may not necessarily mean no damage has been caused. Cheek *et al*. (2001) found fertility and hatchling success were more sensitive to endocrine disruption than vitellogenin expression. This suggests that although no vitellogenin detection occurred in the adult fish collected during this

experiment, there is serious potential for future generations of the same species to be effects. As has been outlined, embryonic and larval stages are the most susceptible to genetic damage, deformations and mortality. If the adults caught during this experiment had been spawning throughout the course of the oil spill, there is a much higher potential for the future generations of these fish populations to be effected. With future generations being compromised, the potential for population-wide collapse or, at the very least, much slower recovery from fishing effort could become a reality in the near future. All fish collected were from the theorized contamination regions of the oil spill, the Gulf of Mexico and the Florida Straits. The techniques and processing procedures could be used in any region where an oil spill and dispersant chemicals could interact with large pelagic fishes.

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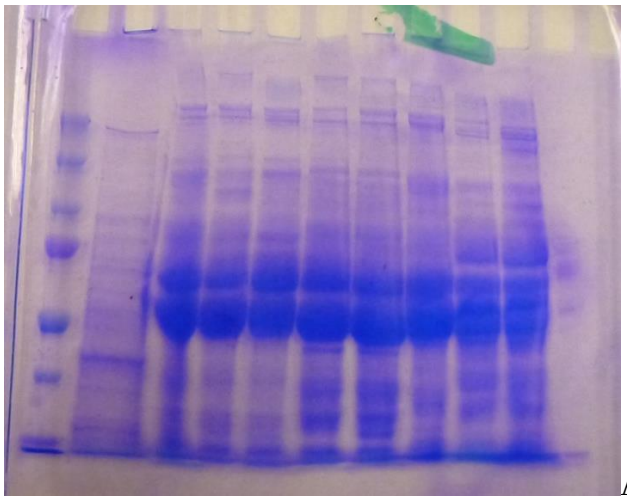
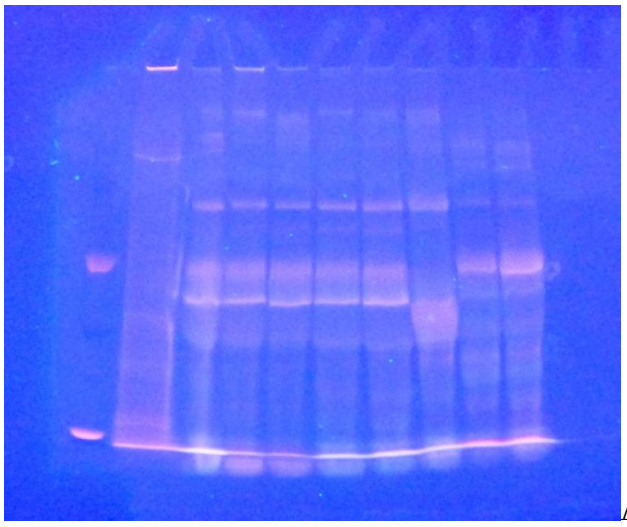
Appendix 1

Gel Photos

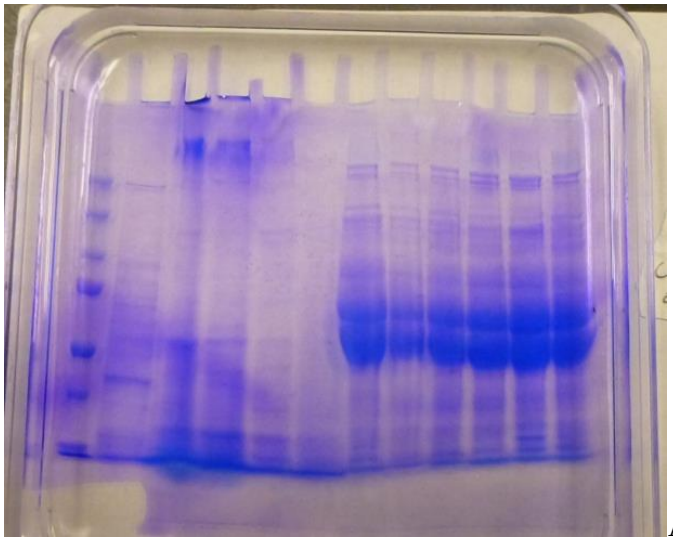

Appendix 1(a).


Appendix 1(b).

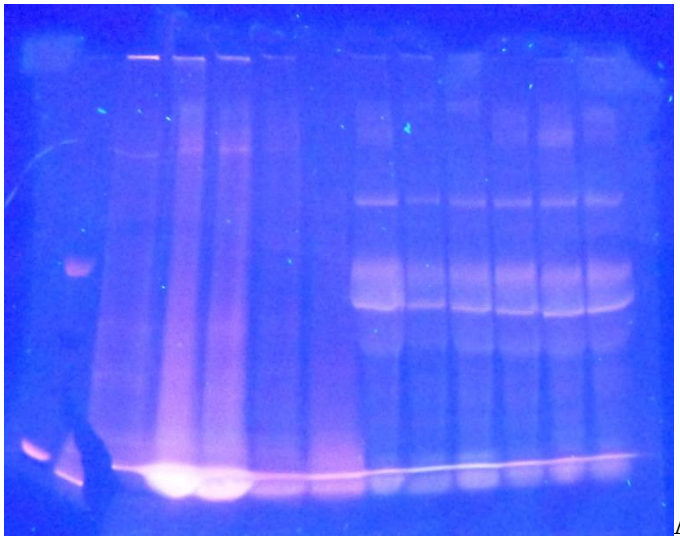
Appendix 1: gel pair 1 illustrates protein banding for one ladder (Precision Plus Protein™ standard – Bio-Rad Laboratories, Inc., Hercules, CA), one control-injected mucus sample from a crevalle jack, three yellowfin tuna mucus samples, one bigeye tuna blood plasma sample, one bigeye tuna mucus sample, one little tunny blood plasma sample, two wahoo blood plasma samples, and two empty wells to the far right. The top image 1(a) is a gel processed with Coomassie Brilliant Blue R-250 gel stain (Bio-Rad Laboratories, Inc.,

Hercules, CA). The bottom image 1(b) is a gel processed with Pro-Q Diamond® gel stain (Life Technologies®, Grand Island, NY).

Appendix 2



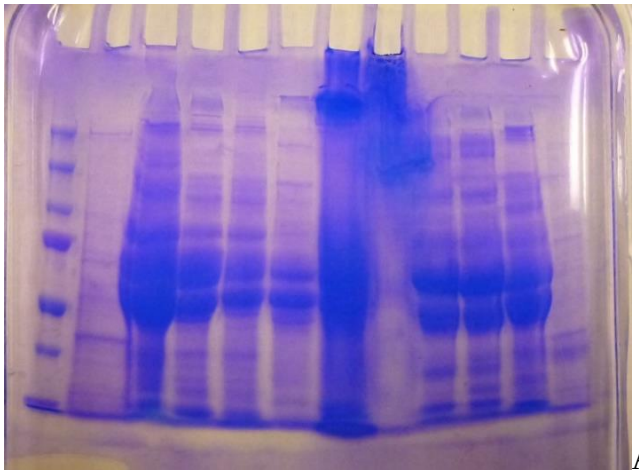
Appendix 2(a).



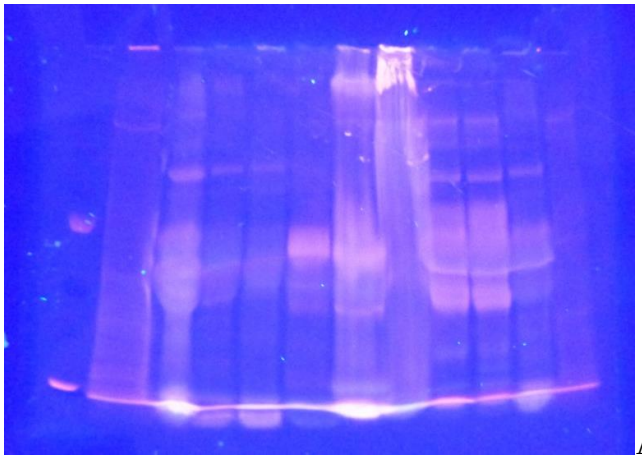
Appendix 2(b).

Appendix 2: gel pair 2 illustrates protein banding for one ladder (Precision Plus Protein™ standard – Bio-Rad Laboratories, Inc., Hercules, CA), one control-injected mucus sample from a crevalle jack, four yellowfin tuna mucus samples, and six yellowfin tuna blood plasma samples. The top image 2(a) is a gel processed with Coomassie Brilliant Blue R-250 gel stain (Bio-Rad Laboratories, Inc., Hercules, CA). The bottom image 2(b) is a gel processed with Pro-Q Diamond® gel stain (Life Technologies®, Grand Island, NY).

Appendix 3



Appendix 3(a).



Appendix 3(b).

Appendix 3: gel pair 3 illustrates protein banding for one ladder (Precision Plus Protein™ standard – Bio-Rad Laboratories, Inc., Hercules, CA), one control-injected mucus sample from a crevalle jack, three blackfin tuna blood plasma samples, 1 skipjack tuna blood plasma sample, two swordfish blood plasma samples*, 2 common dolphinfish blood plasma samples, one yellowfin tuna blood plasma samples and one control-injected mucus sample. The top image 3(a) is a gel processed with Coomassie Brilliant Blue R-250 gel stain (Bio-Rad Laboratories, Inc., Hercules, CA). The bottom image 3(b) is a gel processed with Pro-Q Diamond® gel stain (Life Technologies®, Grand Island, NY).

*Note: the two swordfish blood plasma samples did not exhibit proper protein banding

(indicating sample degradation) during the final processing. Therefore, these two samples are not included in the final sample set.